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Award Number: DAMD17-97-1-7288

TITLE: Dual-Specificity Anti-HER-2/neu Antisense DNA Agents for Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Stanley Stein, Ph.D.

CONTRACTING ORGANIZATION: University of Medicine and Dentistry of New Jersey
Piscataway, New Jersey 08854

REPORT DATE: July 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
Dual-Specificity Anti-HER-2/neu Antisense DNA Agents for Breast Cancer Therapy

5. FUNDING NUMBERS
DAMD17-97-1-7288

6. AUTHOR(S)
Stanley Stein, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Medicine and Dentistry of New Jersey
Piscataway, New Jersey 08854

E-MAIL:
stein@mbcl.rutgers.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We are developing bifunctional agents to reduce HER-2/neu overexpression in breast cancer based on a design coupling an "active" site antisense DNA to a "binding" element. The active site is a DNA hexamer which targets a loop region in the 5'-untranslated region (UTR) of HER-2/neu mRNA. We produced chimeric antisense agents consisting of this active site linked to a binding 2'-O-methy RNA hexamer targeted to another loop region and optimized the linkage between those sites for RNaseH digestion of the target RNA. Cyclic peptide binding moieties have also been optimized. Experiments are in progress to test the efficacy of the new agents in reducing HER-2/neu expression in vitro and in cultured cells.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
27

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

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Stanley Stein/Michael J. Leibowitz 8/30/00

PI - Signature Date

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INTRODUCTION

HER-2/neu, encoded by the erbB-2 gene, is a member of the epidermal growth factor receptor family whose high level of expression in some breast cancers and other malignancies correlates with poor prognosis (Hynes and Stern, 1994; Reese and Slamon, 1997). Inhibition of HER2/neu expression by the therapeutic antibody Herceptin has proven to be clinically useful in treating breast cancer (Ross and Fletcher, 1999). An antisense agent capable of down-regulating expression of this gene should be useful in treating these cancers. Antisense DNA agents can theoretically act by various mechanisms, but appear to exert most of their effect by hybridization to their RNA targets, resulting in the destruction of these targets by cellular RNaseH. As a new approach to the design of antisense agents with high affinity and specificity for the 5'-untranslated region (UTR) of HER-2/neu mRNA, we have studied bifunctional reagents. These reagents consist of a short "active" DNA oligonucleotide binding to a sequence in the target UTR, coupled to a "binding element" which is a chemical moiety with affinity for another portion of the UTR. The most active short DNA oligonucleotide tested was targeted to a predicted single stranded region of the UTR. We have produced bifunctional agents in which attachment of the binding element markedly stimulates RNaseH sensitization at the site targeted by the active oligonucleotide. Experiments are in progress to test the ability of these bifunctional agents to enter cells and to down-regulate HER-2/neu.

BODY

Creation of a peptide through combinatorial synthesis that binds to HER-2/neu RNA

We have previously studied small peptides that bind specifically to RNA regulatory elements, with binding depending on RNA structure (Wang et al., 1995; Choudhury et al., 1998, 1999). To develop the binding element of the bifunctional agent, one approach has been to prepare small libraries conformationally constrained cyclic peptides were prepared on SPOTs membranes, to determine which peptide has optimized specificity of binding to the 5'-UTR of HER-2/neu mRNA. SPOTs membranes are cellulose based and provide a platform for the construction of custom peptide libraries. The cyclic peptide design, shown in Figure 1, provides 6 positions that were varied in the synthesis by adding synthetic (not natural) amino acids whose abbreviations and structures are shown in Figure 2. The amino acids in all six variable positions in the starting peptide to be deconvoluted were D-Arg. The binding assay was performed in PBS by using 10 ng of 5'-[^{32}P]-H HER2 mRNA consisting of 165 bases of the 5'-UTR region in the presence of 10 mg of competitor yeast RNA.

The initial peptide library was deconvoluted at positions X1 and Y1. The SPOTs membrane was placed in a phosphor imager to determine relative affinities of each peptide to HER-2/neu mRNA. The position on the membrane which has the most intense spot corresponds to the spot on the membrane with the greatest amount of radioactivity, and hence the greatest amount of HER-2/neu mRNA which has bound to a peptide. Binding of HER-2/neu mRNA to a given peptide on the membrane was expressed as percent of RNA bound. Results in Table 1 show that the peptide containing L-hLys in position Y1 and Ala-3-pyr in position X1 exhibited the second strongest affinity for the HER-2/neu mRNA among those tested.

A new peptide library was constructed by fixing positions X1 and Y1 and varying amino acids in the X2 and Y2 positions. The binding study was repeated using the same concentrations of both HER-2/neu mRNA and yeast RNA. Table 2 shows the results of the binding assay. The strongest binding peptide containing Ala-4-pip in the Y2 position and Gly-4-pip in the X2 position was used as the fixed amino acids in the third deconvoluted peptide library.

The third peptide library was constructed varying the positions X3 and Y3. The binding study was repeated using the third peptide library and the results of the binding study are shown in Table 3. The optimal peptide for binding specificity by this assay is shown in Figure 3. Deconvolution cycles could be continued at each position using the same or different amino acids. Further studies are planned to attach a HER-2/neu antisense DNA to the best peptide and test the effect of this peptide on binding to the target RNA and on inhibition of HER-2/neu expression in a model system.

HER-2/neu sequence analysis

Our goal is to target the 5' untranslated region of the HER2/neu mRNA, and to perform in vitro and in vivo studies with antisense oligonucleotides to develop agents capable of decreasing in expression of HER-2/neu in cancer cells. In order to generate RNA for in vitro studies, we amplified the 5' end of the HER-2/neu gene. Although multiple studies have yielded

the same sequence for the ORF region of the HER-2/neu gene, five studies have reported four different sequences for the 5' UTR encoded by that gene (Child et al., 1999; Hudson et al., 1990; Coussens et al., 1985; Tal et al., 1987; Yamamoto et al., 1986).

The HER-2/neu sequence was first reported by Coussens et al. (1985; GeneBank accession number M11730). The sequenced DNA was derived from an isolated λgt10 human genomic cDNA clone detected by Southern blot hybridization with oligonucleotide probes for the transforming gene of the avian erythroblastosis virus, v-erbB. This sequence does not contain the entire 5' untranslated region of the HER2 gene, but only 150 bases of the 178 bases that constitute the entire 5' untranslated region, and diverges from the sequence reported by Child et al. (1999) at base -96 (with the A of the translation initiating AUG-Set at +1).

The sequence with GeneBank accession number X03363 (Yamamoto et al., 1986) was derived from an isolated breast cancer cell line cDNA library and detected by probing with a DNA fragment prepared from a c-erbB-2 genomic clone. This sequence contains 174 of the 178 bases that constitute the entire 5' untranslated region of the HER2 gene. The sequence diverges from the sequence of Child et al. (1999) position 141.

Later erbB-2 work focused on the overexpression of the HER-2/neu gene in breast cancer cells. Tal et al. (1987) verified the location of the HER-2/neu promoter region by sequence analysis of genomic and cDNA clones, and by nuclease S1 protection analysis. The promoter region was isolated by screening a λn2 human genomic library, with fragments from HER-2/neu cDNA with the sequence of the isolated clone was reported to (GeneBank under the accession number M16789). This sequence is 757 bases long and contains both the 5' untranslated region and the upstream promoter region of the HER-2/neu gene. This sequence matches the sequence of Child et al. (1999) in the 5' untranslated region, but diverges in the promoter region.

Hudson et al. (1990; GeneBank accession number J05264) reported a sequence derived from a genomic library from human white blood cells, screened with synthetic oligonucleotides representing three known sequences in the upstream region of the c-erbB-2 region. This sequence is 1.4 kb long contains the entire 5' untranslated region of the HER2 gene plus the promoter regions upstream of the transcription start site. The sequence J05264 matches the M16789 sequence and our sequence for the entire 5' untranslated region but diverges 51 bases upstream from the transcription start site.

We determined the sequence of PCR amplified human female genomic DNA (Promega), using PCR primers described by Child et al. (1999). The upstream primer was 5'-TTAAGGATCCGTGGAGGAGGAGGGCTGCTT-3' (labeled as solid arrows, Figure 4), and the downstream primer, 5'-GGGCAAGAGGGCGA GGAG-3', corresponds to the open reading frame of exon 1. The resulting PCR product was then sequenced via an automated DNA sequencer. The results are shown in Figure 4. The same downstream ORF primer failed to amplify DNA from the same template preparation when used in combination with the upstream primers indicated in dashed arrows. (Figure 4,) which are derived from other reported divergent 5' UTR sequences.

Amplified PCR products of the 5' untranslated region were also achieved using primers derived from sequence J05264. We have also confirmed that this sequence is present in the SK-BR-3 cell line, which is derived from a HER-2/neu overexpressing human breast cancer.

Determination of HER-2/neu mRNA Structure

In order to identify possible loops in the nascent HER-2/neu RNA, the 5' untranslated region of the HER-2/neu mRNA sequence was subjected to the Wisconsin GCG mfold software program. This program determines possible 3-dimensional structures of RNA based on predicted energy levels. Figure 5 shows one such 3-dimensional folding of the HER-2/neu mRNA. Single stranded regions are predicted to be the best binding sites for antisense DNA (Ho et al., 1996; Wrzesinski et al., 2000). In order to determine whether these loops do indeed exist, oligonucleotides were constructed that were complementary to the putative loops provided by the mfold program. DNA hexamers were chosen to target the putative loops in the mRNA structure, labeled in yellow. The 5'-[^{32}P]-end-labeled HER-2/neu mRNA consisting of the first 165 bases of the UTR was subjected to RNaseH digestion in the presence of a set of hexamer antisense oligonucleotides complementary to specific regions of the UTR.

The results of this RNaseH sensitization assay are shown in Figure 6. DNA hexamers complementary to loops 1 and 5 failed to sensitize their targets to RNaseH, suggesting that these putative loops did not exist or were not accessible in that region of the HER-2/neu RNA. Hexamers (1 μM and 10 μM) complementary to loops 2, 3, 4, and 6 did sensitize the RNA to RNaseH, confirming that these loops do exist in that region of the HER-2/neu RNA. A hexamer complementary to a stem region of the 5' UTR of the HER-2/neu mRNA did not result in sensitization of the HER-2/neu mRNA to RNaseH. The relative strength of each hexamer to induce cleavage by RNaseH was determined by decreasing the concentrations of the hexamer used in the RNaseH assay. The results in Figure 7 show that the hexamer complementary to loop 3 resulted in the most cleavage of the HER-2/neu mRNA at 0.1 μM and 1 μM as indicated by production of the cleaved RNA fragment and disappearance of the precursor RNA.

Enhanced Sensitivity of HER-2/neu mRNA to RNaseH by Chimeric Oligonucleotides

As an alternative approach to developing low molecular weight antisense agents specific for the 5'-UTR of HER-2/neu mRNA, we synthesized bifunctional agents coupling an active site DNA antisense hexamer with a 2'-O-methyl RNA antisense hexamer acting as a binding ("anchor") moiety. The 2'-O-methyl RNA structure was selected because such antisense molecules bind to their target even more tightly than do DNA molecules, but they fail to sensitize the target to RNaseH and they themselves are not sensitive to DNases or RNases. It has previously been shown that two tethered oligonucleotides binding to nearby targets on an RNA molecule can act synergistically to enhance binding affinity provided the two binding elements are linked in the appropriate geometric arrangement (Moses et al., 1997). Therefore, we tested a series of chimeric antisense molecules against the 5'-UTR of HER-2/neu. These chimeras consisted of a DNA hexamer (active site) targeted to loop 2, and a 2'-O-methyl RNA hexamer (binding site) targeted to loop 3, linked by various linker molecules.

In our first study in this series, two chimeric molecules were used in an in vitro RNaseH assay to determine the sensitivity of the HER-2/neu mRNA molecule to RNaseH relative to that of the hexamers alone. The chimeric molecules consist of two hexameric oligonucleotides chemically linked to each other and oriented in a 5' to 3' direction. The 5' end of the chimeric molecule consists of a 2'-O-methyl RNA hexamer complementary to loop 3 in the HER-2/neu mRNA and the 3' end of the molecule is a DNA hexamer which is complementary to loop 2. Varying lengths of spacers consisting of repeating units of nine carbons in length attach the two hexamers linking the 3' end of the 2'-O-methyl RNA to the 5' end of the DNA hexamer. In this experiment, two chimeric molecules were constructed differing in the number of spacers adjoining the two oligonucleotides, one 9-carbon spacer (Chimera 1) and three 9-carbon spacers (Chimera 3), respectively. The 5'-[^{32}P]-labeled HER-2/neu mRNA was subject to RNaseH digestion in the presence of the chimeric molecules, and the extent of cleavage of HER-2 RNA was compared to reactions containing equal concentrations of the DNA hexamer complementary to loop 2. The results in Figure 8 show that chimeric molecules with both linkers dramatically increased the sensitivity of HER2 mRNA to RNaseH at all concentrations tested compared to the DNA hexamer alone. Significant cleavage was observed for both chimeric molecules at 0.01μM and 0.001μM, with the chimeric molecule containing three 9-carbon spacers exhibiting greater sensitization to RNaseH then did the chimera with only one spacer. The DNA hexamer alone at 0.01μM and 0.001μM failed to cause cleavage of the RNA molecule. The chimeric molecule containing one spacer resulted in a slight increase at another site distinct from the target site. This is not true for the chimeric molecule with three spacers. It appears from these results that the chimeric molecules show increased ability to sensitize the HER-2/neu mRNA to RNaseH mediated cleavage, with cleavage occurring preferentially for the targeted loop.

Sensitization of HER-2/neu mRNA to RNaseH cleavage is dependent on the length of the carbon spacers on the chimeric antisense molecule

In order to further determine which linker length would result in the greatest sensitization of HER-2/neu mRNA to RNaseH activity, chimeric antisense molecules containing 0-4 spacers (Chimeras 0-4) linking the two components were compared. The results in Figure 9 show that the chimeric molecules of all lengths sensitized HER-2/neu mRNA to RNaseH cleavage at all of the concentrations tested. The degree of HER-2/neu mRNA sensitization to RNaseH activity was dependent on the size of the spacer between the two antisense oligonucleotides. Chimeric molecules either lacking a spacer or containing three spacers resulted in the greatest sensitization of HER-2/neu mRNA to RNaseH activity at a concentration of 1nM, as indicated by production of the cleaved RNA fragment and by disappearance of the precursor RNA. Chimeric antisense molecules of all spacer sizes tested enhanced HER-2/neu mRNA sensitization to RNaseH when compared to the DNA hexamer alone. Further studies will examine specificity of the chimeric molecules in the presence of competitor RNA.

Key Research Accomplishments

- Production of a synthetic cyclic peptide that binds specifically to HER-2/neu mRNA
- Production of short antisense oligonucleotides that sensitize HER-2/neu to enzymatic cleavage by RNaseH
- Production of chimeric oligonucleotides that greatly enhance sensitization of HER-2/neu mRNA to enzymatic cleavage by RnaseH

Note: A patent application is in preparation on the chimeric antisense agents.

REPORTABLE OUTCOMES

1. Abstract Presentation - Poster Assignment: CC-38

   Era of Hope
   Department of Defense Breast Cancer Research Program Meeting
   Atlanta Georgia

   Abstract Title: DUAL-SPECIFICITY ANTI-HER-2/NEU ANTISENSE DNA AGENTS FOR BREAST CANCER THERAPY

   Authors: M. J. Leibowitz, J. Perlman, Q. Ding and S. Stein

2. Papers
   A manuscript is in preparation describing the chimeric antisense agent targeting HER-2/neu, which will be submitted after the patent is filed.

3. Patent
   A patent application is in preparation on the chimeric antisense agent targeting HER-2/neu mRNA.

4. Degrees None.

CONCLUSIONS

Sequence analysis and comparison of the 5' untranslated region of the HER-2/neu gene revealed a discrepancy among previously published sequences. This discrepancy could be explained in several ways. The different published sequences might be the result of a genetic rearrangements in the 5' untranslated region of the HER-2 gene, and such genetic rearrangements are common in various cancer cells. It is also possible that this region represents a highly polymorphic region of the human genome. Further PCR experiments applying the various upstream primers to a series of different cancer and human genomic cell lines will determine whether, indeed, this difference is a human polymorphic region or a genetic rearrangement. However, we have attempted to identify other regions of the human genome from which translocated DNA might have been brought into proximity with the erbB-2 gene by computerized searching, and have failed to find such sequences.

Through combinatorial chemistry, we have been able to construct synthetic peptides that vary significantly in their binding to HER-2/neu mRNA. Further deconvolutions should produce peptides that will be able to selectively bind to HER-2/neu mRNA with greater affinity. However, our initial bifunctional antisense molecules were made by another strategy, not utilizing peptides.

We were able to produce short antisense oligonucleotides that are able to sensitize HER-2/neu mRNA to enzymatic cleavage by RNaseH. These short hexameric oligonucleotides take advantage of the unique 3-dimensional structure of RNA molecules and are targeted to single-stranded regions of the HER-2/neu mRNA. Combining these short DNA hexamers with either the synthetic peptide or combining the hexamer to another modified oligonucleotide, we should be able to specifically target the HER-2/neu mRNA and selectively degrade HER-2/neu mRNA in human cancer cells, resulting in lower HER-2 receptor levels. This approach utilizes both an "active" and an "anchor" moiety in antisense drug design.

The 2'-O-methyl RNA hexamer serves as the anchor, and is complementary to loop 3 in the HER-2/neu mRNA molecule. Both the length of the spacer and the chimeric nature of the antisense molecule contribute to the specificity to a given RNA target.

Future studies regarding the cyclic peptide will focus on optimizing its ability to selectively bind HER-2/neu mRNA, including studies with peptides not bound to membranes.

The chimeric antisense molecules based on DNA and 2'-O-methyl RNA hexamers will be the primary thrust of our continuing research. This approach to the construction of a bifunctional agent has yielded impressive data thus far. Further studies will be focused on testing the selectivity of the chimeric molecules for HER-2/neu mRNA. Cell free translation studies are now underway in order to test the effectiveness of this approach on the ability of these molecules to repress translation of the HER-2/neu mRNA. These in vitro will be followed by testing the ability of our new antisense agents to reduced HER-2 expression in various human breast cancer cell lines, which vary in their degree of HER-2/neu overexpression.

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CAPTIONS

Table 1. Binding results of the first deconvolution with cyclic peptides on SPOTs membrane. Positions X1 and Y1 were deconvoluted using synthetic amino acids shown in Fig. 2. The amino acids listed along the top the table refer to amino acids in the Y1 position and amino acids listed down the table refer to amino acids in the X1 position. The other positions were held constant and contained D-Arg. Binding assays were conducted in PBS at 37°C for 2 hours. The spots on the membrane were measured for HER-2/neu binding and expressed as percent of HER-2/neu RNA bound. The peptide exhibiting the strongest affinity for HER-2/neu contained ala-3-pyr in the X1 position and homo-Lys in the Y1 position. These two positions were fixed during the second deconvolution.

Table 2. Binding results of the second deconvolution with cyclic peptides. Positions X1 and Y1 were fixed with the amino acids selected as described in Table 1 while positions X2 and Y2 varied. Binding assay conditions were the same as the earlier binding assay.

Table 3. Binding results of the third deconvolution with cyclic peptides. Positions X1, X2, Y1, and Y2 were fixed as described in Tables 1 and 2, while positions X3 and Y3 varied.

Figure 1. Schematic representation of the cyclic peptides. As indicated in the text and Tables 1-3, cyclic peptides were deconvoluted at the positions labeled X1 through X3 and Y1 through Y3. Construction of cyclic peptides was performed on the SPOTs membrane.

Figure 2. Schematic representation of the synthetic amino acids used in the deconvolution process of reiterative synthesis and screening of cyclic peptides binding to HER-2/neu mRNA.

Figure 3. Schematic representation of the cyclic peptide exhibiting the strongest affinity for HER-2/neu RNA.

Figure 4. Comparison of reported HER-2/neu DNA sequences. Here capital letters indicated sequences identical with the sequence described by Child et al. (1999), and lower case letters indicate sequences diverging from that sequence. Solid arrows indicate primers that resulted in successful PCR amplification from normal human genomic and breast cancer cell line DNA. Dashed arrows indicate primers that did not yield PCR products. PCR was performed with a conserved downstream primer derived from the ORF in exon 1, which was identical in all reported sequences.

Figure 5. Structure of the 5' untranslated region of the HER-2/neu mRNA. The 165 base sequence was subjected to the Wisconsin GCG software mfold program. Schematic representation of the folded structure shown in the figure is based on predicted energy levels of base pairing to generate this structure. DNA hexamers were constructed complementary to the possible loops and stem regions, highlighted in yellow

Figure 6. RNaseH sensitive regions of the 5' region of HER-2/neu mRNA. DNA hexamers complementary to the possible loops and a stem region in the HER-2/neu mRNA structure (see Fig. 5) were hybridized to the 5'-labeled RNA, which was subjected to digestion with RNaseH and analyzed by polyacrylamide gel electrophoresis in the presence of 8 M urea. Untreated RNA and RNA treated with RNaseH in the absence of oligonucleotide are also indicated.

Figure 7. Comparison of the affinity of antisense hexamers for RNA. The experimental procedure was as described in Fig. 6, except the indicated lower concentrations of antisense hexamers were used.

Figure 8. Enhanced sensitivity of 5' region of mRNA to RNaseH by antisense chimeras. The experiment is as described in Fig. 6. Chimera 1 had a single 9-carbon linker and Chimera 3 had 3 linkers between the "active" and "binding" hexamers.

Figure 9. Comparison of different chimeras for RNaseH sensitization of HER-2/neu mRNA. The procedures were as in Fig. 6, where Chimeras 0, 1, 2, 3 and 4 had the corresponding number of 9-carbon linkers connecting the "active" and "binding" hexamers.

Peptide Library 1

X \ Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.4	0.7	1.6	1.7	1.7	1.5	1.4	0.7
2-ABZ-OH	1.3	0.4	1.5	1.4	1.4	1.4	1.7	1.1
Homo-Lys	1.7	0.9	1.4	1.5	1.5	1.3	1.5	1.3
Ala-4-pip	1.7	0.7	1.2	1.3	1.3	2.1	2.2	1.4
L-m-Tyr	1.8	0.6	1.4	1.5	1.2	1.7	1.9	1.8
LTyr(Bn, 3-Cl)	2.5	1	1.8	2.2	1.7	0.9	2.3	1.8
Gly-4-pip	1.7	1.1	2	1.6	1.6	2.3	2	1.6
Ala-3-pyr	1.8	1	**2.6**	2.9	2.2	2.1	2.2	1.6

Selected: X_1=Ala-3-pyr
Y_1=Homo-Lys

Table 1

Peptide Library 2

X \ Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.3	1.1	1	1.1	0.9	1.8	2.1	2
2-ABZ-OH	1.7	1.2	1.7	1.3	1	1.2	1.4	1.6
Homo-Lys	1.9	1.7	2	2	2.1	2.2	1.8	2.2
Ala-4-pip	2	1.8	1.9	2	2.1	1.9	1.9	2.3
L-m-Tyr	1	0.5	0.5	0.7	1.3	1.4	2	1.7
L-Tyr(Bn, 3-Cl)	0.6	0.6	0.7	0.7	0.9	1.5	1.5	1.4
Gly-4-pip	2.2	2.1	2.4	**2.9**	2.7	1.8	1.9	2
Ala-3-pyr	1.8	1.6	1.6	1.9	1.8	1.4	1.5	1.4

Selected: X2= gly-4-pip
Y2=Ala-4-pip

Table 2

Peptide Library 3

X \ Y	D-Lys	2-ABZ-OH	Homo-Lys	Ala-4-pip	L-m-Tyr	L-Tyr(Bn, 3-Cl)	Gly-4-pip	Ala-3-pyr
D-Lys	1.1	1	2.4	**3**	1.9	2.7	2.9	3.2
2-ABZ-OH	1	0.5	1.6	1.8	0.6	1.5	1.6	2
hLys	0.9	0.9	2	3	2.1	2.6	2.2	2.9
Ala-4-pip	1	1.1	1.7	2.7	1.8	1.6	2.3	**3**
L-m-Tyr	0.4	0.2	0.8	1	0.4	0.3	0.5	0.7
L-Tyr(Bn, 3-Cl)	0.6	0.7	0.4	1.2	0.4	0	0.3	0.1
Gly-4-pip	2.2	1.4	1.7	1.6	1	1	1.4	1
Ala-3-pyr	2.1	1.6	1.9	1.5	0.9	1.6	2.1	1.3

Selected: X3=D-Lys
Y3=Ala-4-pip

Table 3

STRUCTURE OF STARTING PEPTIDE

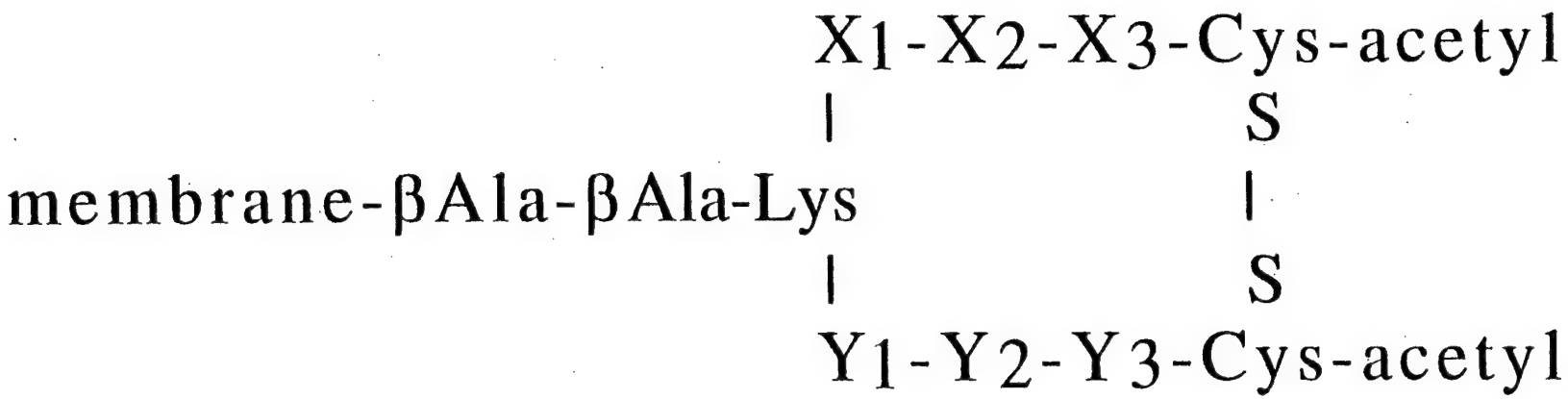
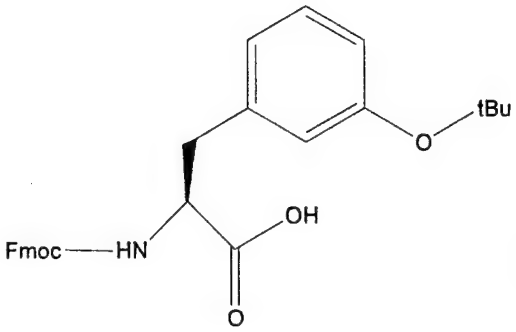
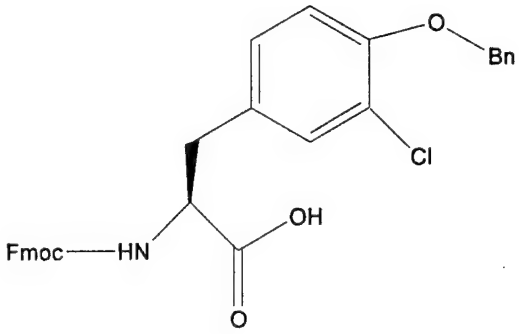


Figure 1

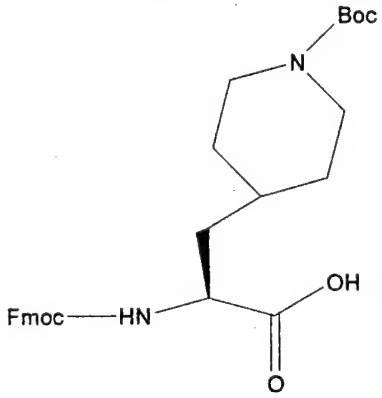
Amino Acid Analogues for Synthesis of Peptide Library



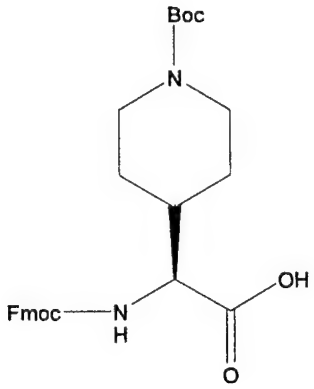
Fmoc-L-m-Tyr(tBu)



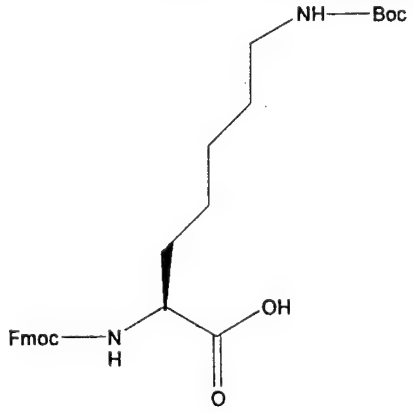
Fmoc-L-Tyr(Bn, 3-Cl)



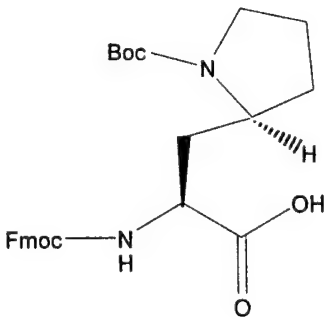
Fmoc-L-Ala-4Pip(Boc)



Fmoc-L-Gly-4Pip(Boc)



Fmoc-L-hLys(Boc)



Fmoc-L-Ala(3-pyrolidinyl-(2-N-Boc)

Figure 2

STRUCTURE OF BEST PEPTIDE

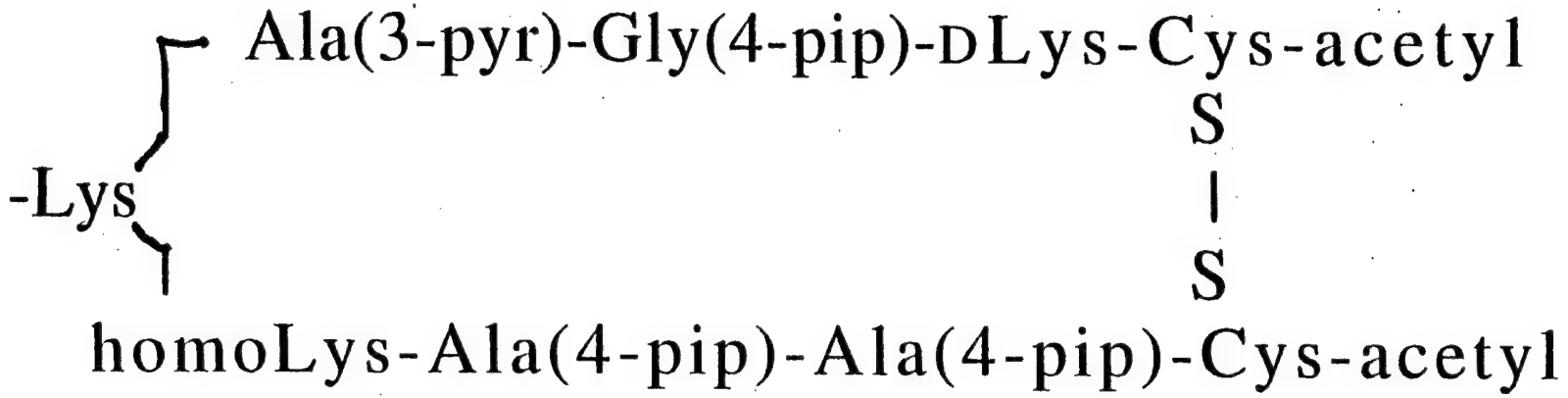


Figure 3

HER2/neu Sequence Comparison of 5' Untranslated End

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                1        10        20        30        40        50        60        70        80        90
Child , et al.  TTAAGGATCCGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCCCCTCCATTGGGACCGGAGAAAC
m16789          gaaggaggagGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCCCCTCCATTGGGACCGGAGAAAC
j05264          gaaggaggagGTGGAGGAGGAGGGCTGCTTGAGGAAGTATAAGAATGAAGTTGTGAAGCTGAGATTCCCCTCCATTGGGACCGGAGAAAC
x03363          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~aaggggaggtaaccctggcccct
m11730          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                91       100       110       120       130       140       150       160       170       180
Child , et al.  CAGGGGAGCCCCCGGGCAGCCGCGCGCCCCTTCCCACGGGGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGC
m16789          CAGGGGAGCCCCCGGGCAGCCGCGCGCCCCTTCCCACGGGGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGC
j05264          CAGGGGAGCCCCCGGGCAGCCGCGCGCCCCTTCCCACGGGGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGC
x00363          ttggtcggggCCCCGGGCAGCCGCGCGCCCCTTCCCACGGGGCCCTTTACTGCGCCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGC
m11730          ~aattctcgagctcgtcgaccggtcgacgagctcgagggtcgacgagctcgagggCGCGCGCCCGGCCCCCACCCCTCGCAGCACCCCGC

                181      190       200       210       220       230       240       250
Child , et al.  GCCCCGCGCCCTCCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
m16789          GCCCCGCGCCCTCCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
j05264          GCCCCGCGCCCTCCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
x03363          GCCCCGCGCCCTCCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
m11730          GCCCCGCGCCCTCCCAGCCGGGTCCAGCCGGAGCCATGGGGCCGGAGCCGCAGTGAGCACCATGGAGCTGG
```

Figure 4

Targets of Antisense Hexamers on 5' Untranslated Region of HER2/neu mRNA

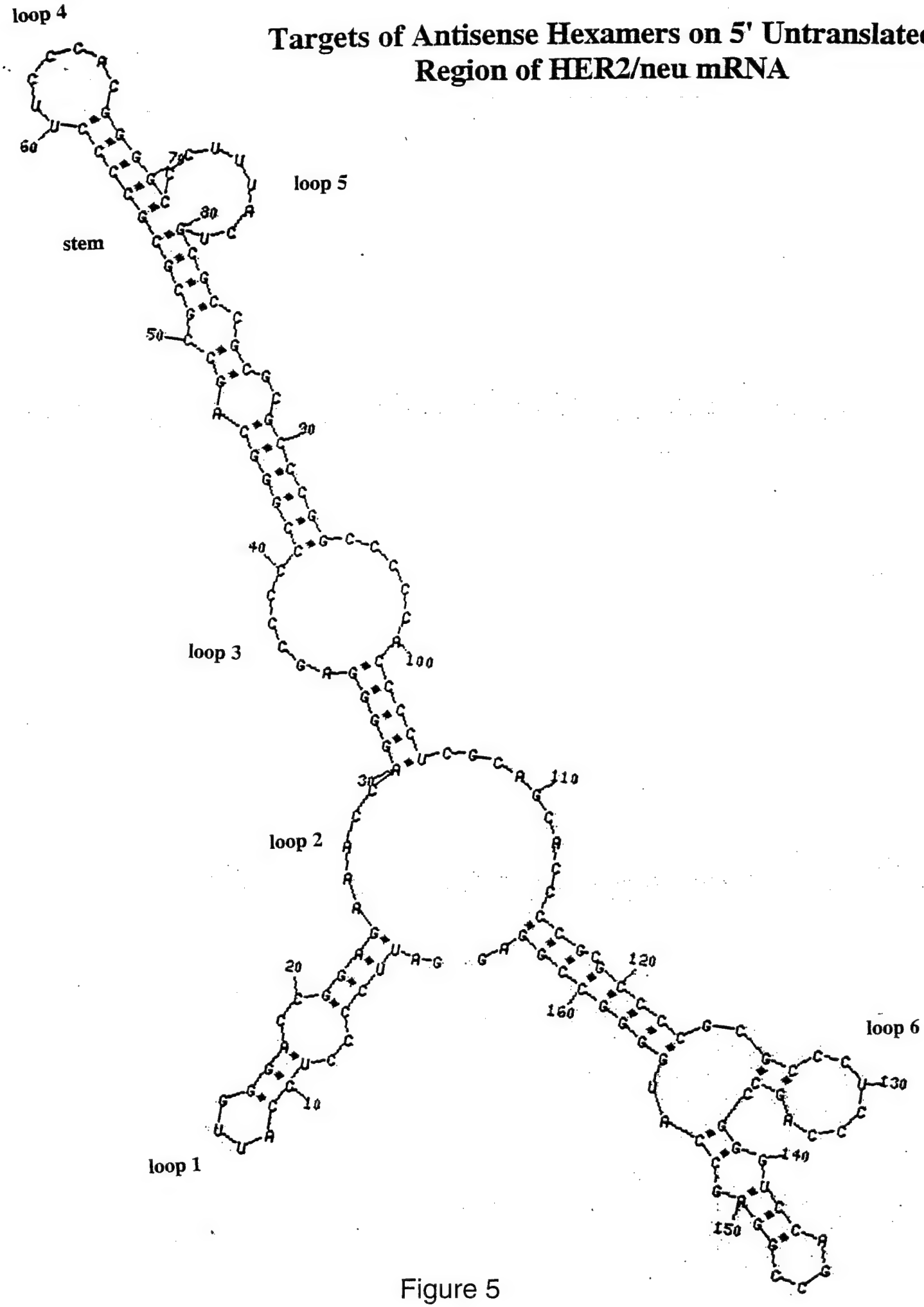

Figure 5

Comparison of RNaseH Sensitization of 5' Region of mRNA by Antisense Hexamers

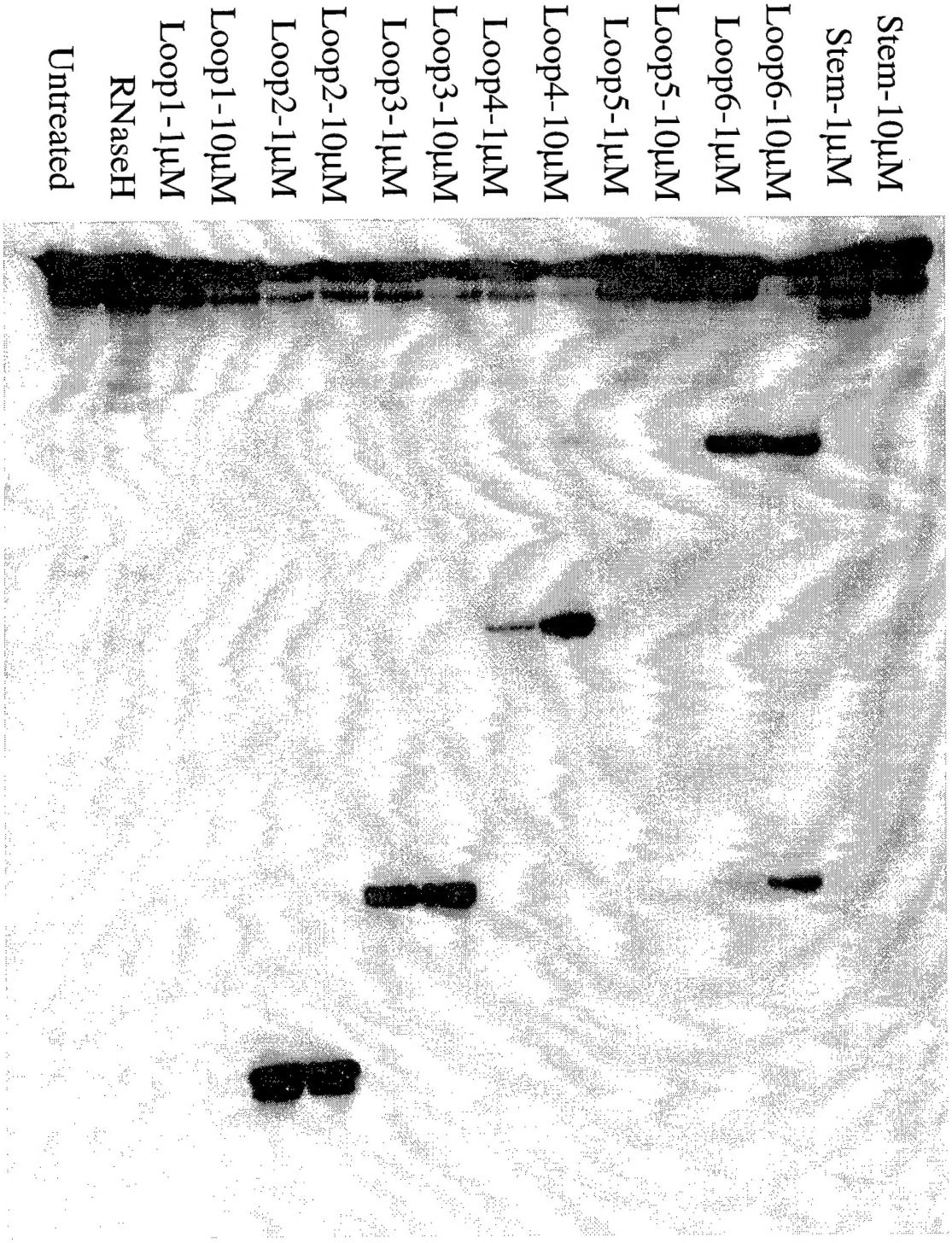

Figure 6

Loop 3 is the Most Sensitive Target for Antisense Binding

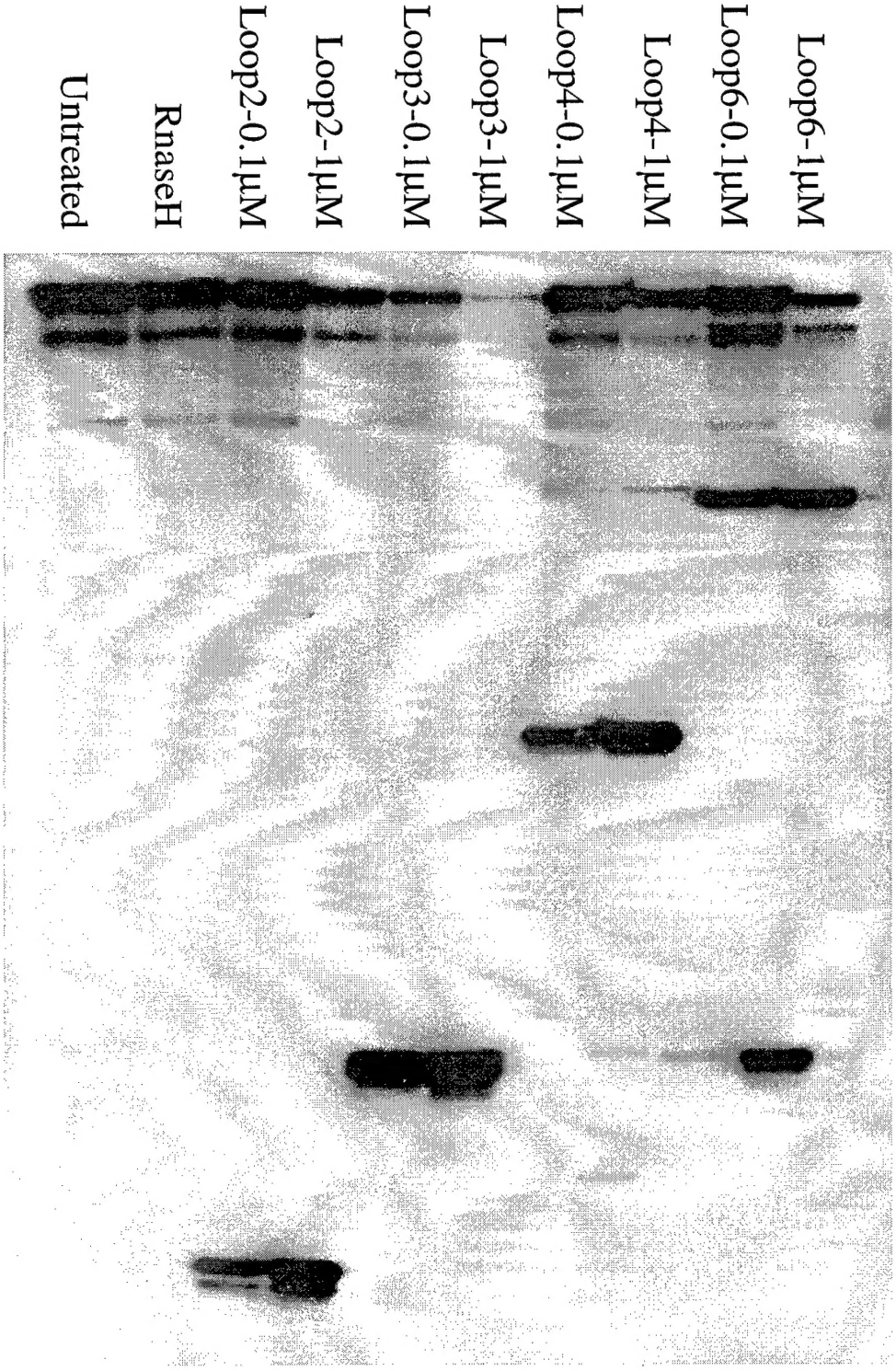

Figure 7

Enhanced Sensitivity of 5' region of mRNA to RNaseH by Antisense Chimera

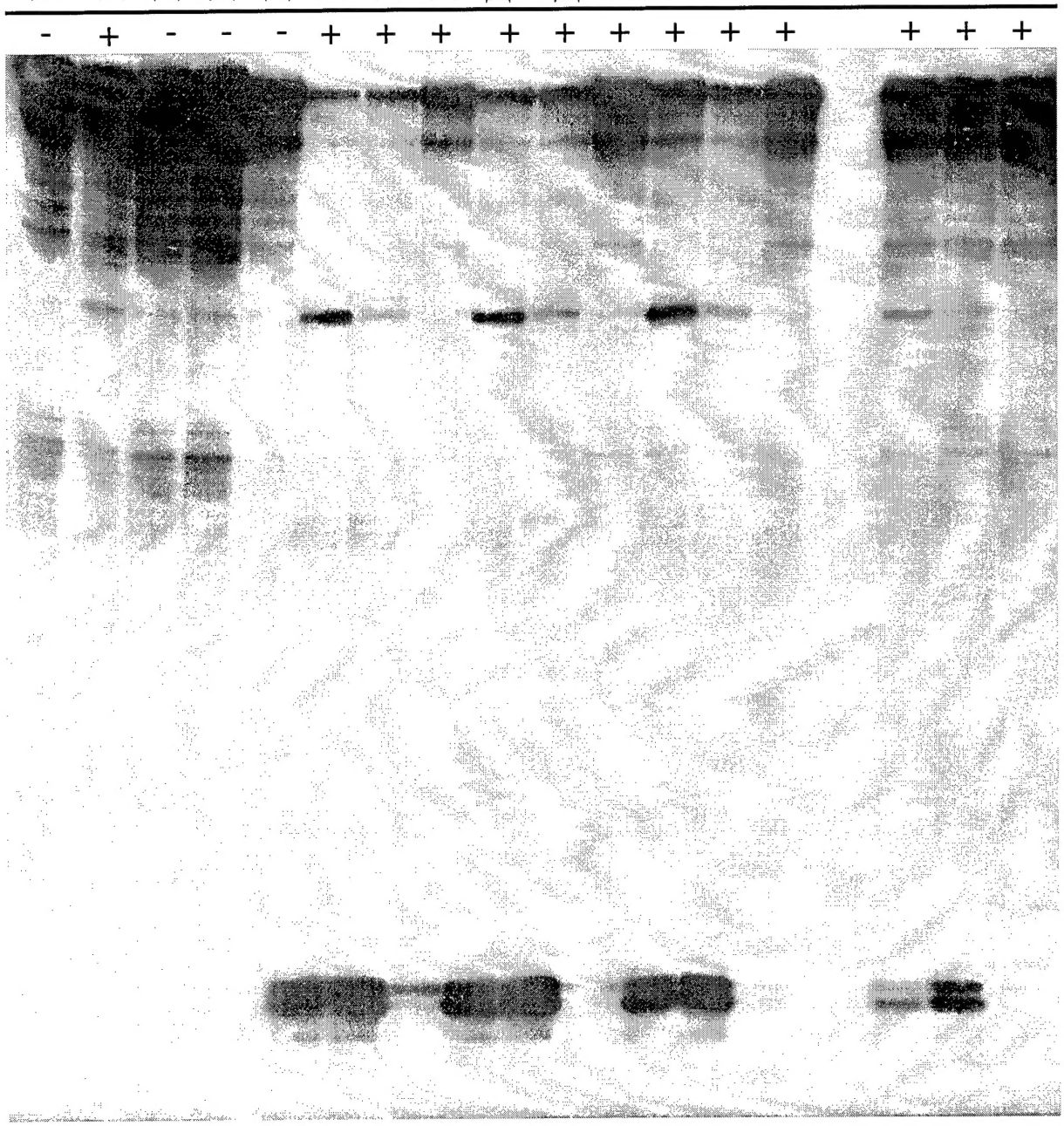

Figure 8

Chimeric molecules containing no spacers and 3 carbon 9 spacers most sensitize 5' region of HER-2/neu mRNA to RNase H

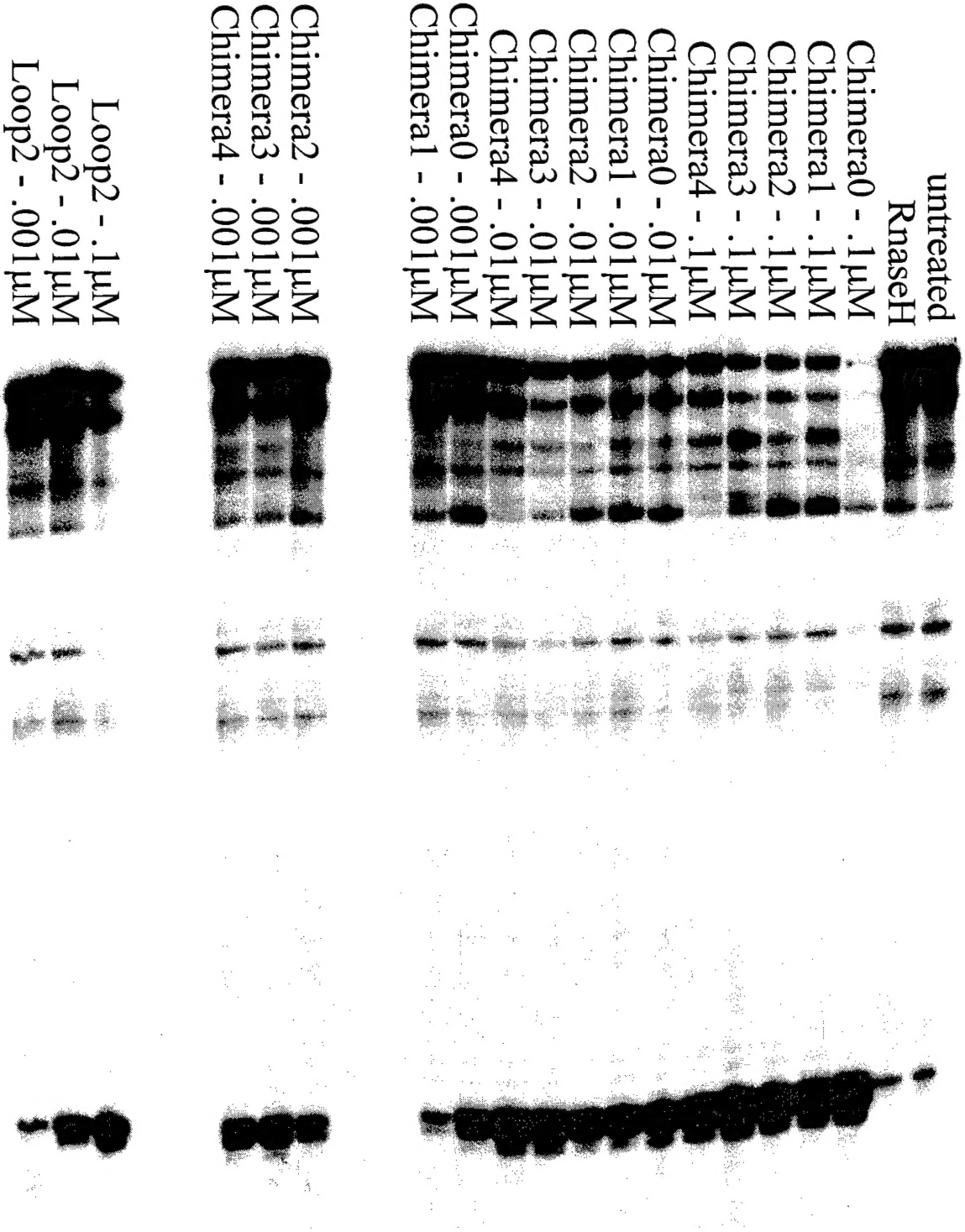

Figure 9